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DOI: https://doi.org/10.1111/bjd.14727

Received Date : 26-Jan-2016

Revised Date : 11-Apr-2016

Accepted Date : 03-May-2016

Article type : Original Article

An exploratory study investigating the metabolic activity and local cytokine profile in melanoma patients treated with pazopanib and paclitaxel

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Running head: Metabolic activity and local cytokine profile under pazopanib and paclitaxel therapy

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bjd.14727

ABSTRACT

BACKGROUND: There is a medical need for new drugs in BRAF wildtype metastatic melanoma patients. Pazopanib is a multi-target tyrosine kinase inhibitor (TKI) with anti-tumour and anti-angiogenic activity.

OBJECTIVES: The primary aim of the study was to investigate the metabolic response to pazopanib monotherapy and pazopanib plus paclitaxel therapy in BRAF wildtype melanoma patients. Secondary endpoints were the early cytokine and chemokine profile and the histological findings.

METHODS: Orally given pazopanib (400 mg twice a day) was administered from day 1 to 10 and from day 14 to day 70. An intravenous infusion with paclitaxel (150mg/m^2 body surface) was administered on days 14, 35 and 56. Metabolic response evaluation was performed before treatment, after treatment with pazopanib (day 10) and after treatment with pazopanib and paclitaxel (day 70). Skin biopsy of metastasis tissue for chemokine and cytokine expression analysis, histology and immunohistochemistry (CD68, CD163) evaluation and blood samples were taken at the same time points.

RESULTS: 2 patients failed screening, 17 were dosed. Out of 67 adverse events, 9 (13%) were grade 3 or 4. Five of 14 evaluable patients had a partial metabolic response at day 10 under pazopanib monotherapy. Response rate at day 70 under combined pazopanib-paclitaxel treatment was 0%. Immunohistochemistry revealed an increase of M2-like macrophages in non-responders compared to responders. We observed a significant upregulation of 5 cytokines in responding (CXCL1, CXCL2, CXCL13, CCL22 and SPP1) versus non-responding lesions. Overall, the median progression-free survival was 70 days (range of 5 to 331 days), which did not significantly differ between responders (148 days) and non-responders (70 days; p=0.17).

CONCLUSION: In this patient population pazopanib efficacy was limited. Response is associated with low M2-like macrophage density and increased expression of several chemokines.

Keywords: melanoma, pazopanib, paclitaxel, cytokine, immune reaction

INTRODUCTION

There has been a dramatic survival benefit in advanced melanoma based on newly developed therapies in the last years including targeted therapies and antibodies against negative modulators of immunological checkpoints [1-4]

For patients with an activating BRAF V600 mutation (estimated prevalence: 40-60% [5]) a BRAF-inhibitor alone or in combination with a MEK inhibitor therapy may be effective [6-8]. In a phase III study, vemurafenib improved the median overall survival significantly in patients with BRAF V600E/K mutations, compared to treatment with darcarbazine [9]. Recent data also show early promising results for NRAS-mutated patients (estimated prevalence: 20-30% [10]) for the treatment with the MEK-inhibitor binimetinib (http://investor.arraybiopharma.com/phoenix.zhtml?c=123810&p=irol-newsArticle&ID=2122959); www.clinicaltrials.gov; Reference number NCT: 01763164) [11,12]. For BRAF and NRAS wildtype patients (i.e. double wildtype patients) there are fewer small molecule pathway inhibitor possibilities. Interference in the mitogen activated protein kinase (MAPK) pathway using single or combined molecular targeted therapies is promising but challenging [13]. A systematic literature review of the multi-target tyrosine kinase inhibitor (TKI) sorafenib did not reveal an explicit benefit in the treatment of melanoma [14]. Sorafenib did not show convincing efficacy in a phase III melanoma clinical trial [15]. However, in a study previously performed in our department, responders to sorafenib showed a clear upregulation of interferon y (IFNy) – stimulated immune response genes in profiled metastases [16].

Pazopanib is an orally-bioavailable, adenosine tri-phosphate (ATP)-competitive tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR) -1, -2, and -3, of the platelet derived growth factor receptor (PDGFR) -α and -β and c-Kit, showing

selectivity for VEGFRs. VEGFR is highly expressed by melanoma cells and is suggested to be associated with disease progression [17]. The VEGF-pathway is a key regulator in angiogenesis. In addition, it modulates the function of T cells, suppressive immune cells and stroma in the tumour microenvironment, leading to an immunosuppressive state [18]. Pazopanib offers the potential to impact on tumour vasculature and the host immune functions. Currently, the drug is approved by the European Medicines Agency for the treatment of renal cell carcinoma and soft tissue sarcoma [19]. It has also shown antitumour activity in ovarian cancer and non-small cell lung carcinoma [20].

Cytotoxic chemotherapy (single agent or combination) has been used for many years without evidence to improve overall survival in advanced melanoma patients. Response rates are typically less than 20% and the median response duration is a maximum of six months [21]. The mean response rate for paclitaxel in advanced melanoma patients as a single agent is estimated to be approximately 17% ([22-24]). Therefore, chemotherapy is currently limited to third line or palliative settings. Nevertheless combination of a cytotoxic chemotherapy with TKI often show superiority to single agent treatment [25].

Fruehauf et al ([26]) performed a clinical trial investigating the combination of pazopanib with paclitaxel for patients with advanced and unresectable metastatic melanoma with promising results. We initiated an open-label, investigator-initiated study for pazopanib-naïve and BRAF wildtype metastatic melanoma patients, with the aim to evaluate the early response to pazopanib. Furthermore, we focused on the cytokine and chemokine profile of the target tumour lesion. We postulate that pazopanib has secondary immunomodulatory properties and influences different aspects of the anti-tumour immune response.

PATIENTS, MATERIALS AND METHODS

Study protocol

The study protocol was developed by RD and JM and approved by the institutional and regional ethical committee (Reference number 2012-0104) and Swissmedic (Authorization number 60326). The trial was registered in www.clinicaltrial.gov (Reference number NCT 01666418). It was conducted in accordance with the ethical principles of the Declaration of Helsinki and in according to good clinical practice.

Patients and Study design

The study was an investigator-initiated, open-label, pilot study performed at the Department of Dermatology, University Hospital Zurich, Switzerland, between June 2012 and December 2013.

Patients with unresectable melanoma stage III and stage IV, according to current AJCC staging system [27], with histologically confirmed skin or lymph node metastases larger than 1 cm in diameter and measurable by Positron Emission Tomography Computed Tomography (PET/CT) scan, were eligible. They were recruited at the Skin Cancer Center of the University Hospital of Zurich. Patients had to be pazopanib-naïve but were allowed to have previous systemic,

targeted, and immune therapies. Patients with significant comorbidities (e.g. severe cardiovascular, gastrointestinal, renal, hepatic or psychiatric conditions, severe infections, metastatic neoplasms other than melanoma or symptomatic metastatic brain or meningeal disease) were excluded. All patients gave written informed consent prior to study inclusion.

Sanger sequencing

Each patient was sequenced for mutations in BRAF exon 15, NRAS exon 2 and 3, and cKIT exon 9, 11, 13, 17 and 18. Primers for each exon are in Supplemental Table 1.

Treatment

The treatment regimen included orally administered pazopanib (Votrient®, GLAXOSMITHKLINE, Switzerland) 400 mg twice a day for 10 days, followed by a drug holiday of 4 days. Pazopanib was restarted at day 14 and given until day 70 (Figure 1A). Intravenous paclitaxel (Taxol®, Bristol-Myers Squibb SA, Switzerland) 150mg/m^2 body surface was given on days 14, 35 and 56. In case a patient showed disease stabilization (tumour regression or stable disease) at the end of the study, the study medication was continued as long as a clinical benefit was observed.

Toxicity

Adverse events were documented according to the National Cancer Institute Common Terminology for Criteria Adverse Events (NCI CTCAE) version 3.0 (www.cancer.gov).

Response evaluation

PET/CT scans with 2-deoxy-2-[fluorine-18]fluoro-D-glucose (FDG) were performed within 28 days prior to baseline and on day 10 and 70 with standardized adjusting. Response to therapy was assessed in PET/CT images using Positron Emission Tomography (PET) response criteria in solid tumours (PERCIST) 1.0 criteria [28,29]. Complete metabolic response (CMR) and partial metabolic response (PMR) were considered as metabolic response; stable metabolic disease (SMD) and progressive metabolic disease (PMD) were evaluated as metabolic non-response.

Blood sample acquisition

Blood samples were collected at day 0, day 10 and day 70 and processed routinely in the laboratory of the University Hospital Zurich. Standard values for lactate dehydrogenase (LDH) and S100B were defined as lower or equal to the reference cut-off of 480 U/l and 0.2 µg/l, respectively.

Biopsy collection

The investigators defined a reference metastasis (subcutaneous or lymph node metastasis, more than 1 centimetre in diameter) prior to the start of the study in every patient. Serial biopsies were taken from the opposite ends of the same tumor if larger than 2 cm or taken from neighbouring comparable metastases. The biopsies were performed before treatment (baseline), after treatment with pazopanib (day 10) and after treatment with pazopanib and paclitaxel (day 70) and preserved in RNAlater and cryopreservation (Ambion, Life Technologies Corp., Carlsbad, CA, USA) for chemokine and cytokine profiling and in formalin for immunohistochemistry.

Histology and immunohistochemistry

All tissues used for immunohistochemistry were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinised in xylene and rehydrated. Haematoxylin and eosin (H&E) staining was used to evaluate the overall morphology of the metastasis and the surrounding tissue. Epitope retrieval was performed in antibody-specific buffers. The following antibodies were used: CD68 (DAKO, Glostrup, Denmark), CD163 (DAKO, Glostrup, Denmark), and CD8 (DAKO, Glostrup, Denmark). Staining was performed using kits supplied by DAKO REAL Detection System (kit 5005). Antigen-specific antibodies were applied and visualized with the ChemMate detection kit (DAKO). Slides were counter-stained with H&E. Staining intensity for CD68 and CD163 was graded by board-certified dermato-pathologists (RD, EG). Macrophage density was graded in 3 levels, level 1 corresponding to lower than 30%, level 2 between 30% and 60% and level 3 to above 60% of total cell count.

Chemokine and cytokine profiling and data analysis

Tissue biopsies were evaluated by a board certified dermatopathologist to identify the tumor region. Biopsies with a minimum of 80% tumor area were submitted for total RNA extraction with the QIAGEN RNAeasy kit (Qiagen, Venlo, Netherlands). RNA purity and quality was determined by Nanodrop, samples were excluded if 260/230 was below 1.5 and 280/260 was below 1.8. cDNA was made from the RT2 HT First Strand Kit (330441; Qiagen) according to manufacturer's instructions. Genes were evaluated with the Human cytokine & chemokine PCR array (PAHS-150ZA; Qiagen) with the Viia7 system from Applied Biosystems. Fold change and P values were calculated by RT2 Profiler PCR Array Analysis (Qiagen). Genes were normalized to the three housekeeping genes on the array.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego California USA). Statistical analysis for immunohistochemistry data was performed using the Mann-Whitney U Test. Comparison of Kaplan-Meier survival curves was performed using log-rank test. P values of less than 0.05 were considered statistically significant.

RESULTS

Patient population

Nineteen patients were enrolled in the study. Two of them did not meet all the inclusion criteria or presented with exclusion criteria. Out of 17 patients included (12 male and 5 female, mean age: 69.8 years ± 12.0 years), 16 (94.1%) were classified as stage IV and 1 patient with amelanotic vulvar melanoma as stage III. One patient presented with asymptomatic brain metastases prior to study inclusion. The detailed patient characteristics are shown in Table 1.

Toxicity to Pazopanib and Paclitaxel

The median treatment duration was 62 days (minimum 5 days, maximum 70 days). 67 adverse events (AEs) of any grade related to the study drugs were reported in 15 of the 17 patients (88% of patient population). The other common AEs occurring during anti-VEGF treatment such as hypertension and proteinuria were not observed in this study ([30-34]). Only one patient presented with epistaxis.

According to grade 1 or 2, 87% of all adverse events (AEs) were mild or moderate and included constitutional symptoms (fatigue, loss of appetite, dysgeusia and weakness), skin changes (alopecia, maculopapular rash), impairment of bone marrow function (anemia, leucopenia and neutropenia), neurological side effects (peripheral dysesthesia) and gastrointestinal side effects (nausea, diarrhea, constipation, elevated liver parameters).

Nine (13%) of all the AEs were classified as serious adverse events (SAEs). The most frequently reported SAEs were impairment of the bone marrow function (3 cases of neutropenia grade 4) and liver toxicity (3 cases of elevated liver parameters grade 3). The other SAEs were severe fatigue, dysgeusia and wound infection.

Adverse drug reactions led to study discontinuation in 8 patients: 2 of them due to serious neutropenia, 1 patient due to fatigue, 1 patient because of pneumonia, 1 as a consequence of persisting dyspnea, 2 due to worsening of the general condition and 1 because of severe dysgeusia.

All adverse events are summarized in Table 2.

Metabolic response at day 10 and day 70 evaluated by PERCIST

Fourteen of the 17 treated patients in our study were amenable to metabolic evaluation of the PET/CT at day 10 (under pazopanib monotherapy) (Figure 1B). Three patients were not evaluable due to early progression. One target lesion was selected for analysis under PERCIST 1.0 criteria (Figure 2A). The mean SUV (Standardized uptake value) at baseline (n=17) was 16,0 (± 9,6), at day 10 (n=14) 13,6 (± 9.5) and at day 70 (n=7) 16,5 (± 11,2). Based on the SUV of the target lesions (day 10 versus day 0), we identified 5 patients with PMR under therapy with pazopanib (Table 3, Figure 2B). The 9 non-responders included 5 patients with SMD and 4 with PMD. Responders under pazopanib had a mean SUV- reduction of 67.6 % at day 10 (± 16.4), while non-responders had a mean SUV-increase of 17% (± 32.8) at this time.

The analysis of the local response at day 70 (under therapy with pazopanib and paclitaxel) compared to baseline was feasible in 8 patients (3 with PMD, 3 with SMD and 2 PMR) (Figure 2C). Local disease control rate (CMR+ PMR+ SMD) at day 10 for the 14 patients with analysable PET/CT came up to 71.4%. Global response rate at day 70 was 0%. Five patients presented SMD and continued the therapy off-study.

Mutation status

In the responder group, we had 3 patients with wildtype status for BRAF and NRAS. One patient had a c-kit mutation and one patient had NRAS mutation. The non-responder group consisted of 6 double-wildtype patients and 3 patients with BRAF wildtype and NRAS mutation.

Progression free survival and overall survival

Median progression-free survival (PFS) was 70 days (range from 5 to 177 days) (Figure 3A). Median PFS in the local responders was longer (148.5 days) than in non-responders but was not significant (70 days), p=0.17.

Median overall survival (OS) was 208 days (range from 69 to 1073 days) (Figures 3B). Median OS in the local responders showed a trend to be longer than in the non-responders, 494 and 208 days respectively, but not significant (p=0.31).

The reason for discontinuation of the study was progression in 9 patients and adverse events in 8 patients.

LDH and S100 (Serum)

Mean LDH value at time of screening (n=16) was 632 U/l ± 244 U/l, at day 10 (n=15) 739 U/l ± 437 U/l and at day 70 (n=8) 581U/l ± 192 U/l. The responder group had lower (not significant) levels of LDH compared to the non-responder group at each time point. (Figure 3C, Table 4).

Mean S100 value at time of screening (n=17) was 3,4 µg/l ± 6,2 µg/l, at day 10 (n=15) 3,0 µg/l ± 6,4 µg/l and at day 70 (n=8) 1,0 µg/l ± 1,22µg/l. The responder group had lower (not significant) levels of S100 compared to the non-responder group at each time point (Figure 3D, Table 4).

Biopsies (Histology)

We analysed haematoxylin-eosin stained slides of the metastases of 4 responder and 4 non-responder patients at day 0 and day 10. The tumour cells presented epitheloid large cell morphology or nested small to medium size morphology. The tumour areas showed a wide variation of melanin content, focal areas of necrotic cells, inflammatory infiltrates and mitotic figures. The intra-individual morphology of the tumour cells was similar. There were no consistent changes in morphology between day 0 and day 10 (Figure 4A).

Biopsies (Immunohistochemistry)

The immunohistochemical stainings of CD68/CD163 (glycoproteins, expressed on monocytes and tissue macrophages) served to estimate macrophage and M2-like macrophage density and distribution in metastatic tissue. CD8 was also evaluated to estimate cytotoxic T- cell infiltration. Non-responders exhibited higher frequencies of CD163 positive cells and CD8 positive cells (day 10 versus baseline) mainly around the tumour stroma (Figure 4B). CD68 showed a comparable staining pattern suggesting that most, if not all CD68+ cells also expressed CD163.

Biopsies (Cytokine and chemokine profiling to pazopanib treatment)

The differential chemokine and cytokine analysis was performed on 3 responders and 3 non-responders as the other 24 samples had Cycle threshold values below the detectable limit. Overall, the responders had the majority of cytokines and chemokines upregulated at day 10 compared to baseline, while the non-responders had the majority of cytokines and chemokines downregulated at day 10 (Figure 5A). There were 5 out of 84 significant differentially expressed cytokines and chemokines between the non-responders and the responders: CXCL1, CXCL2, CXCL13, CCL22, and SPP1 ($p < 0.05$) (Figure 5B). On average, the responders had at least 0.5-fold higher expression of these chemokines at day 10 and the non-responders had at least 2-fold lower expression at day 10.

DISCUSSION

Melanoma patients with BRAF and NRAS wildtype tumours have limited options when treated with targeted small molecule inhibitors. There is a dire need for new therapies for these patients. Multi-target TKIs have not been well established in melanoma patients so far.

PET/CT is a powerful imaging technique that - in contrast to conventional CT - demonstrates metabolic alterations early during a drug intervention. Selective kinase inhibitors such as vemurafenib induce a drastic decrease of glucose uptake [35] within 15 days after treatment initiation. The reduction of SUV after 3-4 weeks might help to identify patients with a prolonged benefit from targeted therapies [36].

Previously, we have used PET/CT to investigate early responses during treatment with the multi kinase inhibitor sorafenib. It was able to identify responders already after ten days. Interestingly, the reduction of SUV was clearly associated with morphological and immunological signs of regression such as increased expression of interferon gamma [16]. These data suggested that tumour regression induced by sorafenib is accompanied by a plethora of immunological processes. However, sorafenib was not successful in randomized clinical trials [14,15,37-39] despite promising early phase results.

Our study was designed to investigate the metabolic response and early local immunological events induced by pazopanib together with paclitaxel.

Pazopanib is a poorly investigated drug in metastatic melanoma. Fruehauf et al. [26] presented data on a phase 2 trial using pazopanib and paclitaxel in 55 melanoma patients. They reported an overall response rate of 33% and a median PFS of 7.7 months. A similar response rate was reported for patients treated in a Phase I dose escalation trial [40]. We did not observe objective responses in our patient population that presented with several prognostic factors associated with poor outcome such as M1c stage, multiple distant metastases and elevated LDH and S100 levels [41].

To evaluate the contribution from the immune system during pazopanib treatment, we stained for CD68, CD163 and CD8. An interesting finding in our study was the higher amount of macrophages in the non-responders compared to the responders and the lack of CD8 cells in the responders but surprisingly the increase in CD8 in the non-responders. Tumour associated macrophages (TAMs) have been described before as tumour promoting by supporting the proliferation, survival and motility of cancers cells [42]. This hypothesis is supported by our immunohistochemical findings for CD68 and CD163. CD68 is a commonly used marker in immunohistochemistry to identify all macrophages [43] and CD163 is used as a marker to identify M2-like macrophages [44]. CD163 positive macrophages have been associated with TAMs [45].

Most CD68 positive cells were also CD163 positive, suggesting a macrophage tumour supportive environment. We observed a higher staining intensity of CD163 positive tumour-associated macrophages in the non-responder group compared to responder group before treatment and at day 10 of pazopanib treatment; however it was not statistically significant due to the lower number of samples. In conjunction with the increase in CD163, CD8 positive cells were also increased. We suspect that the CD163 population is dampening the CD8 response. Though surprisingly, there was no significant increase in CD8 infiltration in the responder group, suggesting a CD8 independent mechanism of response. These results were confirmed in the qPCR results. The vast majority of cytokines and chemokines were upregulated at day 10 in the responders compared to the non-responders. Interferon gamma was upregulated in the responder group (1.0 fold upregulation versus -2.8 fold downregulation), however it was not significant.

The cytokines and chemokines that passed statistical significance were CXCL1, CXCL2, CXCL13, CCL22, and SPP1. CXCL1 and CXCL2 are known to be secreted by macrophages for neutrophil recruitment in the presence of necrotic cells [46,47]. This finding would suggest that pazopanib causes necrotic cell death, which attracts macrophages for neutrophil recruitment to the tumour. An interesting finding is the upregulation of CCL22 and SPP1 in the responder group. CCL22 is a chemokine that is typically secreted by M2 macrophages [48]. CCL22 recruits T-regulatory cells, which dampens the immune response. SPP1 also known as osteopontin has been found to promote melanoma growth, angiogenesis and invasion [49,50]. Osteopontin levels in melanoma patients' serum have been observed to increase with the stage of the disease, with its highest levels at stage IV [51]. These two tumour promoting chemokines might be responsible for the short response duration to pazopanib. The role of CXCL13 in melanoma has not been well described; however, CXCL13 has been implicated in the progression of prostate cancer [52].

Our interpretation of the qPCR and immunohistochemistry results suggests that melanoma cells could be rescued from TKI induced stress conditions by the presence of M2 macrophages. Responding tumours secrete a mix of tumour suppressing and tumour promoting cytokines and chemokines. As there were no responders at the end of the study, it would suggest that the inhibition of tumour promoting cytokines and chemokines could have added benefit.

Therefore, strategies to inhibit tumour-associated macrophages are needed. Today, antibodies suppressing the macrophage population with an anti-M-CSF antibody like MCS110 from Novartis are in clinical trials. Currently, clinical trials with MCS110 in combination with checkpoint inhibitors are in preparation.

Emactuzumab (RG7155) is a monoclonal antibody that inhibits CSF1R activation and was successfully used in patients with pigmented villonodular synovitis, which is a malignant disease driven by macrophages [53]. These molecules should be investigated in combination with multi-kinase inhibitors in order to overcome macrophage mediated tumour cell protection.

In conclusion, pazopanib had limited efficacy in this patient population. M2-like macrophage density was associated with poor response and increased cytokine and chemokine expression was associated with early response.

Disclosure of Potential Conflicts of Interest

R.D. receives research funding from Novartis, Merck Sharp & Dhome (MSD), Bristol-Myers Squibb (BMS), Roche, GlaxoSmithKline (GSK) and has a consultant or advisory board relationship with Novartis, Merck Sharp & Dhome, Bristol-Myers Squibb, Roche, GlaxoSmithKline, Amgen outside the submitted work.

S. M. G. has temporary consultant or advisory relationships and receives travel support from Roche, Novartis, Bristol-Myers Squibb (BMS), Merck Sharp & Dome (MSD); she receives research funding from the University of Zurich and SAKK.

Acknowledgments and Funding

We thank GlaxoSmithKline for supporting this Investigator Induced Trial through educational grant to the University of Zurich. GSK was not involved in the study design, data collection or analysis or manuscript draft. We thank the Clinical Trial Unit for their support, Ines Kleiber-Schaaf for histology and Mirka Schmid for technical assistance.

This work has further been supported by the Swiss National Science Foundation (PMPDP3_151326) and the Forschungskredit of the University of Zurich (FK-14-032).

Statement of Significance

What's already known about this topic?

- Increased expression of VEGF and other tyrosine kinases leads to tumours promotion, associated with poor prognosis in patients with melanoma.
- Pazopanib is a multi-target TKI effective in several tumour types suggested to suppress VEGF effects and has shown some efficacy in melanoma.

What does this study add?

- Pazopanib showed limited activity in advanced melanoma patients
- Responding tumours demonstrate high expression of several chemokines and cytokines
- Resistant tumours show high numbers of tumour associated M2-like macrophages

ClinicalTrials.gov Identifier: NCT 01666418

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TABLES

Table 1: Demographics, tumour characteristics and treatment course of the individual patient.

Pat. Nr.	Gender	Age	AJCC Stage at baseline	TNM stage (according to AJCC staging and classification)	Pre-treatments #	BRAF Status	NRAS Status	Other Mutations	LDH ≥ 480 U/l at baseline *	S100 ≥ 0.2µg/l at baseline **	PERCIST day 10 of reference metastasis	PERCIST day 70 of reference metastasis	PERCIST of all metastases day 70	Time on Treatment (nr. of days)	Reason for discontinuation
1	M	86	IV	M1a	1. DTIC 2. Ipilimumab	wt	wt		Y	Y	SMD			62	AE

2	**F**	**82**	**IV**	**M1c**	**1. DTIC 2. Ipilimumab 3. Vindesine**	**wt**	**wt**		**Y**	**N**	**PMD**	**PMD**	**PMD**	**70**	**PD**
3	F	69	IV	M1c	1. Hyperthermic Isolated Limb Perfusion (TNF, Melphalan)	wt	wt		Y	N				5	AE
4	M	60	IV	M1b		wt	wt		N	N				48	PD
5	**M**	**76**	**IV**	**M1b**		**wt**	**wt**		**N**	**N**	**PMR**			**30**	**AE**
6	M	92	IV	M1b		wt	wt		N	N				15	AE
7	F	72	IV	M1c	1. Temozolomide 2. Ipilimumab	wt	wt		Y	Y	PMR			21	AE
8	M	65	IV	M1c	1. Peginterferon alpha-2b 2. Pasireotide	wt	wt		Y	N	SMD	PMR	SMD	70	AE
9	**M**	**88**	**IV**	**M1b**		**wt**	**wt**	**c-Kit 572_575del DPTQ insE**	**N**	**N**	**PMR**			**37**	**AE**
10	**F**	**53**	**IV**	**M1a**	**1. Ipilimumab 2. MEK 162 + RAF 265**	**wt**	**wt**		**N**	**N**	**PMR**	**SMD**	**SMD**	**177**	**PD**

					3. Temozolomide									
11	M	75	IV	M1c		wt	NRAS Q61R	Y	Y	SMD			45	AE
12	M	56	IV	M1c	1. Adjuvant interferon therapy 2. Ipilimumab 3. Pembrolizumab	wt	NRAS Q61R	Y	Y	SMD	PMR	SMD	174	PD
13	**M**	**61**	**IV**	**M1c**	**1. Interferon alpha 2. DTIC 3. Binimetinib**	**wt**	**NRAS Q61K**	**Y**	**Y**	**PMR**	**PMD**	**SMD**	**120**	**PD**
14	M	59	IV	M1c	1. Binimetinib 2. Ipilimumab	wt	NRAS Q61R	Y	Y	SMD	SMD	SMD	162	PD
15	**M**	**55**	**IV**	**M1c**	**1. Adjuvant Interferon alpha-2b 2. Ipilimumab**	**wt**	**Wt**	**Y**	**Y**	**PMD**			**17**	**AE**
16	**M**	**65**	**IV**	**M1c**		**wt**	**Wt**	**Y**	**N**	**PMD**	**SMD**	**PMD**	**70**	**PD**
17	**F**	**71**	**III**	**IIIC**		**wt**	**wt**		**Y**	**PMD**	**PMD**	**PMD**	**70**	**PD**

Abbreviations:

AJCC, American Joint Committee on Cancer. DTIC, Dacarbazine. wt, wildtype. Y, yes. N, no.

Metabolic response according to PERCIST 1.0 criteria: CMR, Complete Metabolic Response. PMR, Partial Metabolic Response. SMD, Stable Metabolic Disease. PMD, Progressive Metabolic Disease. AE, Adverse Event. PD, Progression of Disease.

Pre-treatment indication is limited to systemic therapies.

* Lactate dehydrogenase (LDH) upper normal range: ≥ 480U/l.

** S100 ≥ 0.2µg/l

Patients in bold: 8 patients further analysed

Table 2: The safety population included 17 patients that received at least one dose of study drug. Listed are all adverse events grade 3 and 4.

Adverse Events in 17 Patients		
Adverse Event	Number of patients	Relationship to study medication
Wound Infection		
Grade 3	1	Related
Grade 4	-	
Pneumonia		
Grade 3	1	Not related *[1]
Grade 4	-	
Dyspnea		
Grade 3	1	Not related *[2]
Grade 4	-	
Delirium		
Grade 3	1	Not related *[3]
Grade 4	1	Not related *[3]
Neutropenia		
Grade 3	-	
Grade 4	3	Related
Fatigue		
Grade 3	1	Related
Grade 4	-	
Elevated Liver parameters		
Grade 3	3	Related

Grade 4	-	
Dysgeusia		
Grade 3	1	Related
Grade 4	-	
Pain		
Grade 3	1	Not related *[1]
Grade 4	-	
*[1] Tumour related *[2] Pre-existing *[3] Related to urinary infection		

Table 3: Changes of SUV value in the reference metastasis during study course. Specification in per cent (%), referring to baseline value.

	Baseline	Day 10	Day 70
Responders	0	-82.1	-96.1
	0	-75.7	
	0	-72.7	
	0	-67.8	
	0	-39.7	106.0
Non-Responders	0	-28.6	-31.3
	0	-26.6	-45.1
	0	-12.0	
	0	5.9	29.7
	0	14.7	
	0	30.4	-7.5
			54.7
	0	37.9	
	0	41.0	
	0	45.3	

Table 4. Lactate Dehydrogenase (LDH) and S100 levels of responders and non-responders.

	Day 0	Day 10	Day 70
Responder (LDH)	(n=5) 497.6 U/l (± 95.1)	(n= 4) 680.2 U/l (± 199)	(n=2) 501.5 U/l (± 231.2)
Non-responder (LDH)	(n=8) 729.0 U/l (± 75.93)	(n=9) 796.3 U/l (±157)	(n=5) 633.0 U/l (± 204.8)
Responder (S100)	(n=5) 1.6 µg/l (± 0.75)	(n=4) 0.3 µg/l (± 0.23)	(n=1) 0.1 µg/l
Non-responder (S100)	(n=9) 4.4 µg/l (± 1.94)	(n=9) 4.5 µg/l (± 2.37)	(n= 5) 1.3 µg/l (± 1.5)

Figure Legends:

Fig. 1A: Study design of the trial. Doses of Pazopanib (400 mg twice a day) were administered from day 1 to 10 and from day 14 to day 70. Intravenous Paclitaxel (150mg/m2 body surface) was given on day 14, 35 and 56. Evaluation of PET/CT (Positron emission tomography-computer tomography), blood and biopsy samples was processed before treatment (baseline), after treatment with pazopanib and after treatment with pazopanib and paclitaxel. In between the two treatment regimens, patient passed through a 4-day washout phase.

Fig. 1B: Flowchart of patient disposition.

Fig. 2A: Representative PET/CTs of a responder and a non-responder patient before and after treatment with pazopanib (day 0, day 10). Non-responder: PET/CT images with disseminated disease, reference metastasis localized in the left lower leg. Responder: PET/CT images with reference metastasis in the left shoulder.

Fig. 2B: Waterfall plot showing the percentage of SUV difference between day 0 and day 10 in target lesion. In red, non-responding patients. In blue, responding patients. Reference bar indicating the 30% reduction of SUV, standing for partial metabolic response (PMR).

Fig. 2C: Spider plot displaying the SUV changes of each target lesion in the course of the study at day 0, day 10 and day 70. 17 patients had a measurement at day 0 and only 8 remained at day 70. 3 patients had PMR, 3 patients with stable metabolic disease (SMD), and 2 patients with progressive metabolic disease (PMD).

Fig. 3A: Progression-free survival of all patients. Black curve: All patients included. Green curve: Responder group. Red curve: Non-responder group. The responders had a median progression free survival time of 149 days and the non-responders had a median progression free survival time of 70 days ($p = 0.17$)

Fig. 3B: Overall survival of all patients. Black curve: All patients included. Green curve: Responder group. Red curve: Non-responder group. The responders had a median survival time of 494 days and the non-responders had a median survival time of 208 days ($p=0.31$).

Fig. 3C: Lactate dehydrogenase (LDH) levels of responders and non-responders through the course of the study. Red boxes: responder group. White boxes: non-responder group.

Fig. 3D: S100 levels of responders and non-responders through the course of the study. Red boxes: responder group. White boxes: non-responder group.

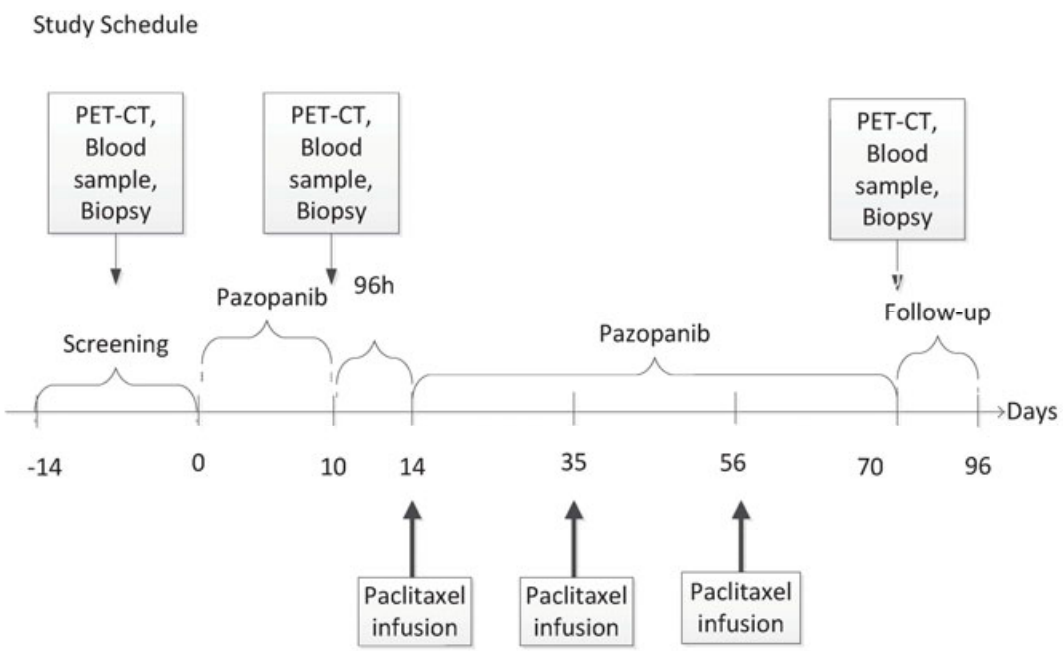
Fig. 4A: Histology and immunohistochemistry of a reference metastasis. First row: H&E (Haematoxylin and eosin) stainings, second row: CD68, third row: CD163, fourth row: CD8. Scale bar = 100μM.

Fig. 4B. Quantification of CD68, CD163, and CD8 stainings of the 8 patients analysed

Fig. 5A: Bar graph representing the 84 chemokine and cytokine expression changes at day 10 after treatment with Pazopanib. Blue bars represent the responder group and red bars represent the non-responder group.

Fig. 5B: Bar graph of the 5 out of 84 significantly upregulated cytokine and chemokines after treatment with Pazopanib.

A



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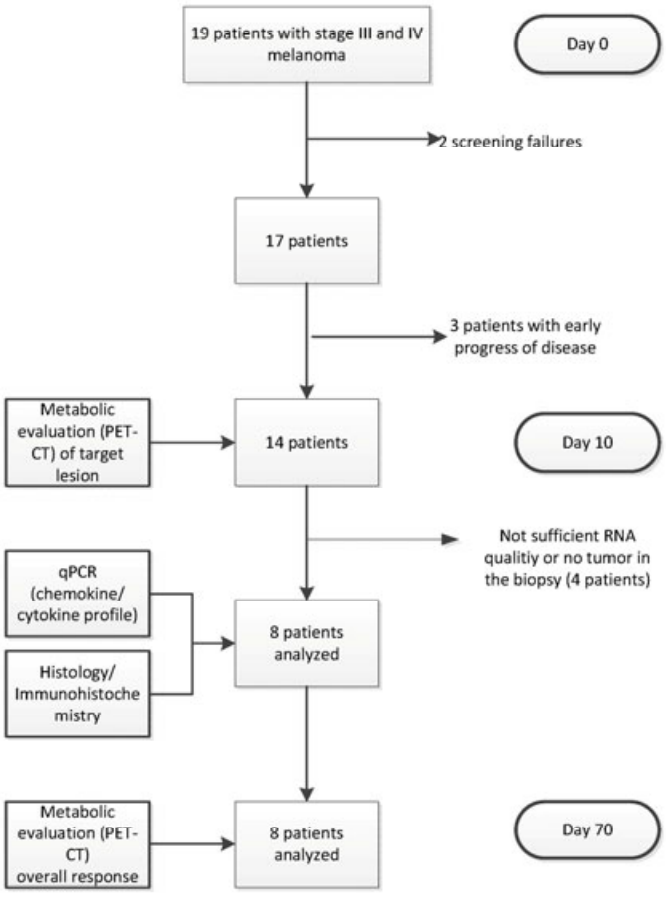

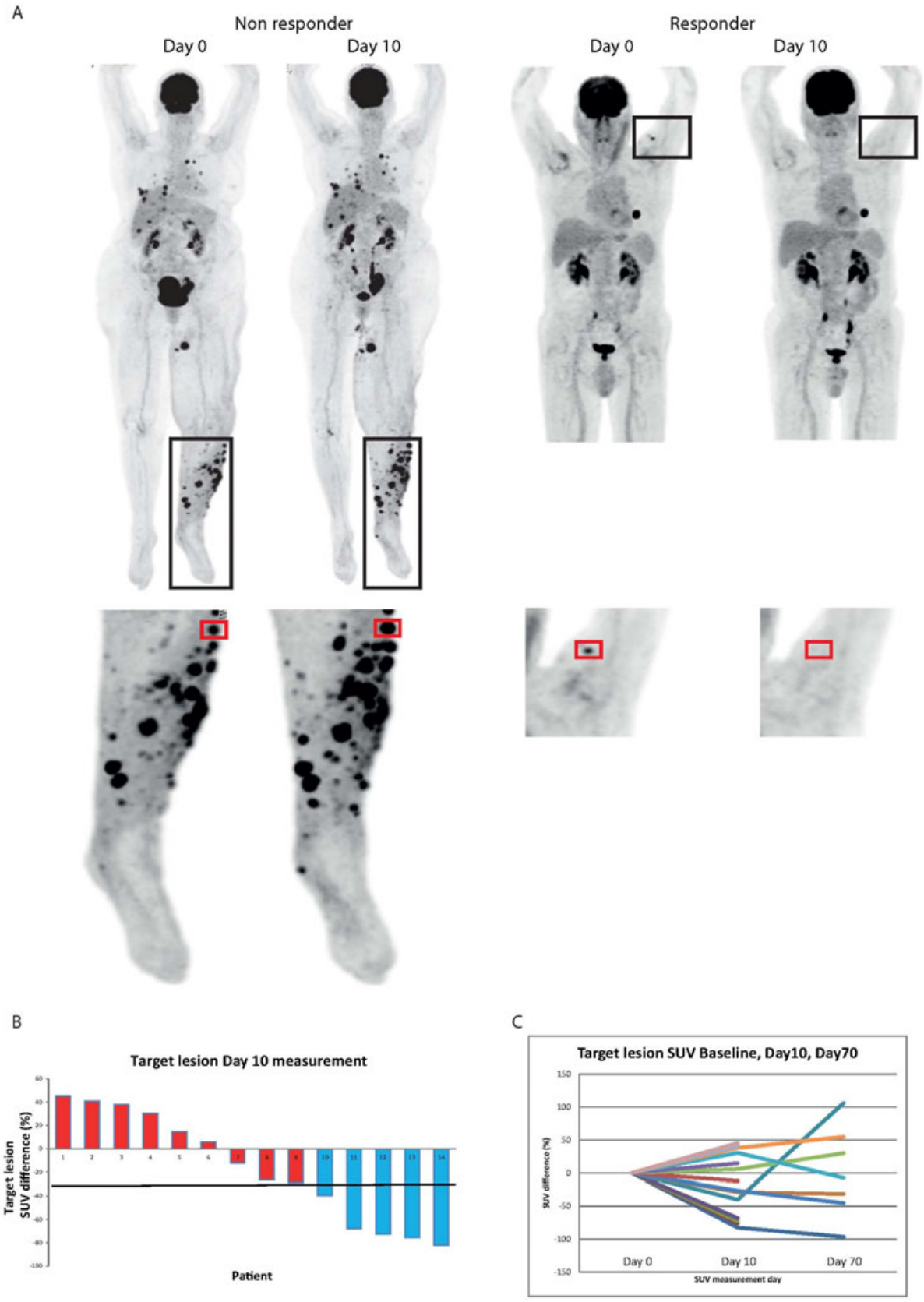

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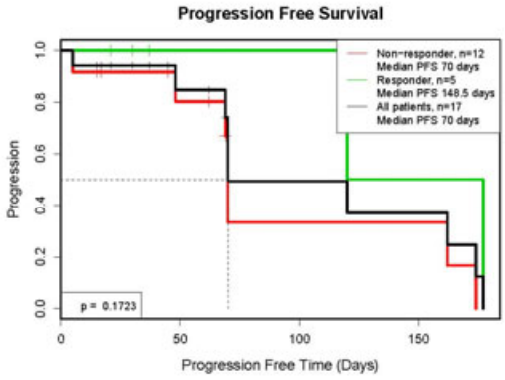

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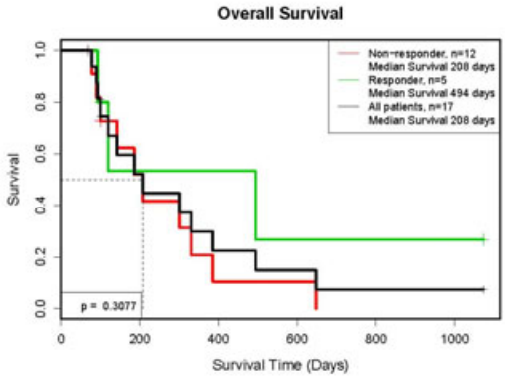

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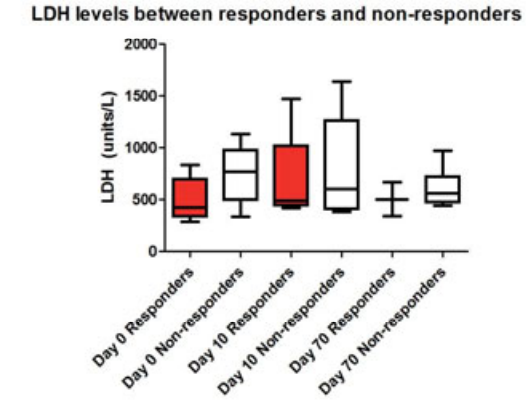

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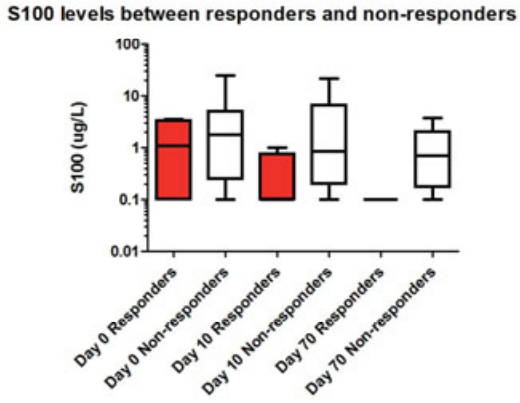

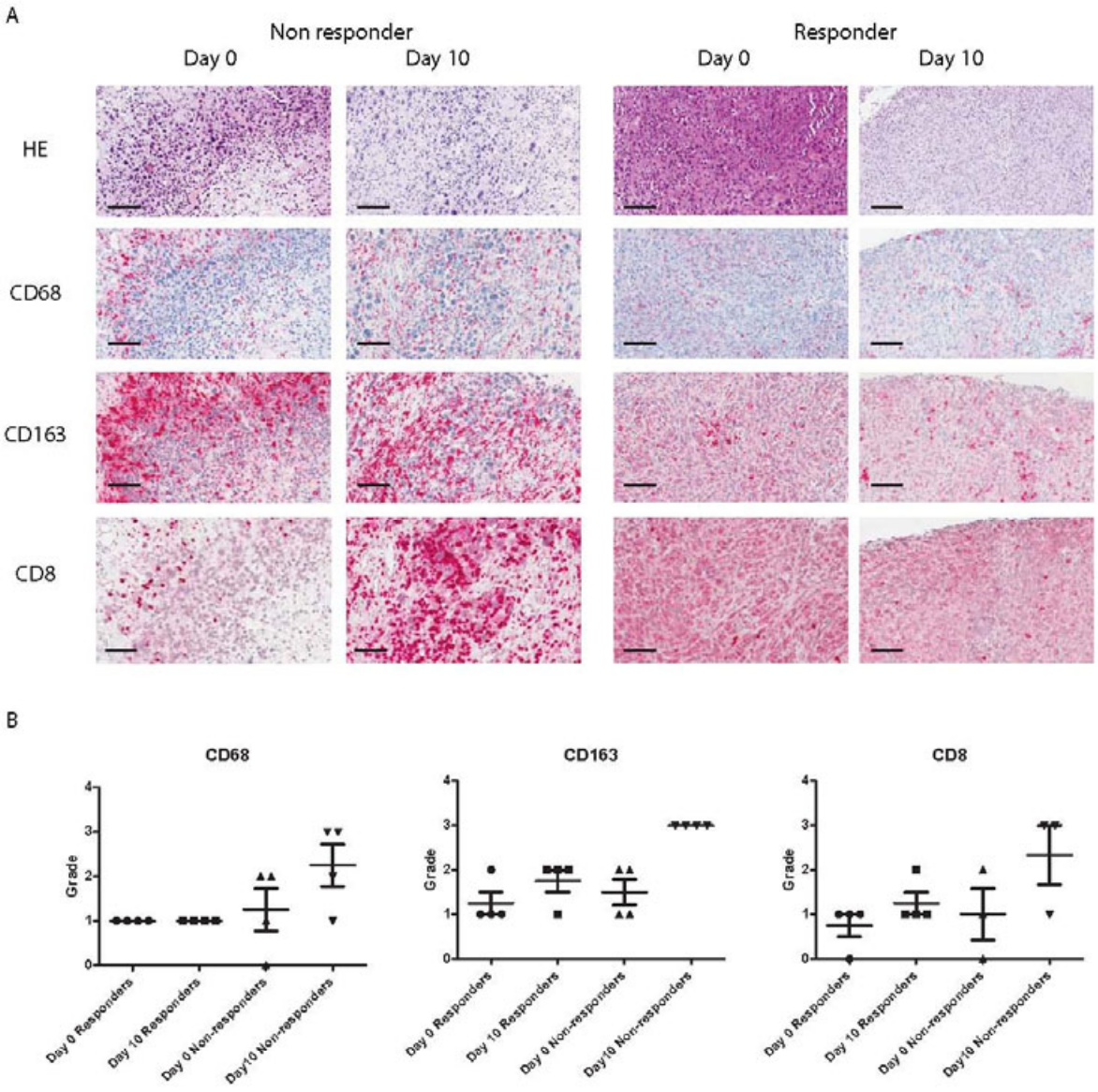

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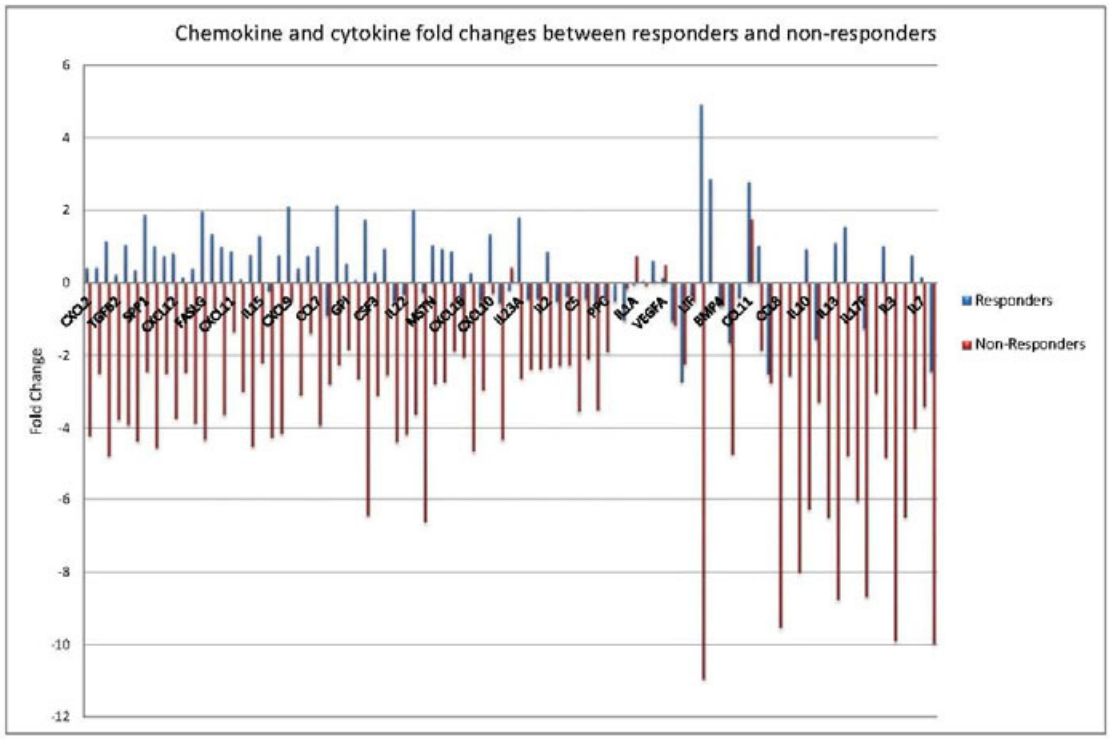

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